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EFFECT OF HYDROSTATIC PRESSURE AND ELECTROPORATION ON BACTERICIDAL EFFICIENCY IN COMBINATION WITH BACTERIOCINS AND LYSOZYME

PHASE I

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13. ABSTRACT *(Maximum 200 words)*
The bactericidal efficiency of ultrahigh hydrostatic pressure (UHP) and pulsed electric field (PEF) were studied in combination with two bacteriocins, nisin A and pediocin AcH, and lysozyme. Several Gram-positive and Gram-negative bacterial species and strains associated with food spoilage and foodborne diseases were tested. The results showed that both UHP and PEF inflict lethal and sublethal injury to Gram-positive and Gram-negative bacterial cells. As the sublethally-injured cells became sensitive to the bactericidal action of the two bacteriocins and lysozyme, the combination of the UHP and PEF treatment, along with bacteriocin and lysozyme resulted in greater viability loss than when only the UHP or PEF are used. In general, under the study conditions, UHP and bacteriocins produced more viability loss than PEF with and without bacteriocins. By combining bacteriocins and lysozyme, UHP at 30,000 psi for 1 min produced viability loss of 13.8 logs in <u>Listeria monocytogenes</u> Scott A and 6.3 logs in Salmonella <u>typhimurium</u> as compared to 2.8 logs for both species by UHP alone and 4.7 and 4.1 logs, respectively, by UHP + bacteriocins.

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PREFACE

This study was conducted during the months of October 1993 through September 1994 by Mssrs. Norasak Kalchayanand and Bibek Ray, University of Wyoming, under the supervision of Mssrs. Anthony Sikes and Patrick Dunne, (ADD) of Sustainability Directorate, U.S. Army Natick Research, Development and Engineering Center, Natick, MA. The work was funded under the project (DJ10) titled "Antimicrobial effectiveness of ultra-high hydrostatic pressure and pulse electric field in combination with bacteriocins for use in food preservation", DJ10:# DAAK60-93-K-0003.

Mssrs. Kalchayanand's and Ray's research was designed to ascertain the following: (1) do UHP or EP treatments to pathogenic and spoilage gram-positive and gram-negative bacterial cells induce sublethal injury; (2) do these sublethally injured cells become more susceptibile to antibacterial peptide of bacteriocins; (3) do UHP or EP treatments in combination with bacteriocins increase viability loss of pathogenic and spoilage bacteria, and (4) does lysozyme, in combination with UHP or EP treatments and bacteriocin, enhance viability loss of these bacteria?

This research, which was divided into 3 phases, was initiated on 1 Oct 93. This report summarizes results from Phase I, which ended 30 Sept 94.

EFFECT OF HYDROSTATIC PRESSURE AND ELECTROPORATION ON BACTERICIDAL EFFICIENCY IN COMBINATION WITH BACTERIOCINS AND LYSOZYME

PHASE I

Introduction

Ultrahigh hydrostatic pressure (UHP) and pulsed electric field (PEF) are being investigated in the USA as possible nonthermal methods of food preservation due to their antimicrobial effectiveness (5, 8, 14, 17 and 18). The UHP can be applied to both solid and liquid or pumpable foods, whereas PEF is effective only in liquid foods. The UHP processed food retains its physical appearances, color, flavor and nutrients. All UHP treatment also causes improvement in the texture of some foods, and destabilization of some undesirable enzymes (3, 4, 5, 14, 17 and 18). Both UHP and PEF destroy microbial cells (vegetative) by destabilizing the structural and functional integrity of the cytoplasmic membrane (2, 10, 11 and 17). The amount of cell death is directly proportional to both the level of pressure and the duration of UHP process and both the voltage and the total pulse time during PEF process (2, 11 and 17).

Antimicrobial peptides or bacteriocins of lactic acid bacteria have been shown to be bactericidal to sublethally injured gram-positive and gram-negative bacteria (12, 22, 23 and 24). Therefore, the hurdle concept of food preservation (16) can be applied to increase the antimicrobial efficiency of UHP and PEF methods by combining with bacteriocin treatment.

Limited studies have revealed that UHP has increased antimicrobial efficiency in combination with low heat treatment, low pH, lysozyme, chitosan or carbon dioxide (4 and 17). The mechanism(s) of this greater microbial destruction has not been explained. It may be due to sublethal injury of cells by the UHP and their susceptibility to these compounds. Many stresses are known to impose sublethal injury to microbial cells, which then become sensitive to different physical and chemical environments (21). We are currently studying the effectiveness of several biopreservatives such as bacteriocins to increase the antimicrobial efficiency of UHP and electroporation (EP, a form of PEF) methods when used in combination.

The objectives of this study were to determine that both UHP and EP treatments produced the following effects: (a) induce sublethal injury on bacterial cells and (b) have increased bactericidal efficiency in combination with bacteriocins and/or lysozyme.

METHODS AND MATERIALS

Bacterial strains and cell preparation

Three pathogens, *Listeria monocytogenes* Scott A, Escherichia *coli* O157:H7 strain 932, and *Salmonella typhimurium* M1, and two spoilage bacteria, *Leuconostoc* strain O3 and *Serratia liquefaciens* Lm, from our culture collection, Animal Science Dept., U. of Wyoming, Laramie, WY, were used. *Leuconostoc* was grown in lactobacilli MRS broth for 16 to 18 h at 30°C and all other bacteria were grown in tryptic soy broth (TSB) supplemented with 0.6% yeast extract for 16 to 18 h at 37°C. The cells were harvested by centrifugation at 7,000xg for 10 min at 4°C. The cells were washed and resuspended to obtain 10^{12} to 10^{14} cells per ml either in 0.1% peptonized water for use in UHP studies or in PM buffer (7 mM sodium phosphate, 1 mM $MgCl_2$ [pH 6.5]) for EP studies (13). Initial studies showed that at a higher pressure, many cells die quickly. A high cell concentration was, therefore, used to facilitate determination of the numbers of injured and dead cells, especially in the combination studies of UHP and bacteriocins. The cell suspensions were maintained at 4°C before and after UHP and EP treatments and prior to enumeration of colony-forming units (CFU).

Enumeration of viable and injured cells

To determine the level of the viable population, a cell suspension was serially diluted and surface plated simultaneously on prepoured plates of a nonselective agar (TSA; Difco, Detroit, MI) supplemented with 0.6% yeast extract and a selective agar (modified Oxford medium for *L. monocytogenes*, violet red bile [Difco, Detroit, MI] for *E. coli*, and xylose-lysine deoxycholate [Difco, Detroit, MI] for *S. typhimurium*). The plates were incubated at 37°C for up to 2 d, and CFU per ml were determined. *Leuconostoc* was enumerated on MRS agar whereas *S. liquefaciens* was enumerated on TSA. Plates were incubated at 30°C for 2 days.

Bacteriocin preparation

Pediocin AcH and nisin were prepared from the broth cultures of *Pediococcus acidilactici* strain LB 42-932 and *Lactococcus lactis* strain 11454 from our culture collection (U. of Wyoming, Laramie, WY), respectively. Preparation, purification, and assay for their activity units (AU) were according to the previously described methods (1 and 27).

Lysozyme preparation

Lysozyme hydrochloride (SPA, Bio SPA Division; purified grade) was dissolved in deionized water at the concentration of 0.04g/ml. The solution was membrane filtered through 0.45 µm low protein binding syringe filter (Gelman Sciences, Ann Arbor, MI) and chilled at 4°C before using.

UHP treatment

Each bacterial suspension was filled to capacity into small plastic vials (Cryovial; Simport Plastic, Quebec, Canada; 2 ml capacity) in duplicate. When necessary, purified pediocin AcH (identical to pediocin PA-1 [9]), nisin, or a mixture containing equal amounts of both was added to the cell suspension to give a final concentration of 5,000 activity units (AU) per ml (27). Lysozyme was added at the concentration of 100 µg/ml. For a comparison, 1 International Unit (IU) of nisin was found to be equivalent to 100 Activity Units (AU) of our preparation of either nisin or pediocin AcH (19). The vials were individually put in plastic bags and vacuum sealed. Then the vials were put into the chamber (6 by 18 in.; 15.24 by 45.72 cm) of the hydrostatic pressure unit (Harwood Engineering, Walpole, MA). Liquid (oil) was pumped into the chamber until the desired pressure (30,000 or 50,000 lb/in^2) was reached, held for the desired time (approximately 1 min), and then released to drop the pressure to atmospheric pressure (14.7 lb/in^2). Since the pump was controlled manually, the times to attain a particular end pressure from atmospheric pressure and to drop back to atmospheric pressure differed between experiments (Table 2). The temperature of the liquid remained almost unchanged at room temperature (22°C) at high pressures. The vials were removed and stored at 4°C, and CFU per ml were enumerated within 2 h.

PEF treatment

The cell suspensions were subjected to EP in a Gene Pulser Unit (Bio-Rad Laboratories, Richmond, CA.). Cell suspensions (200 µl) were placed in cuvettes (0.2 cm). When required, purified bacteriocins and/or lysozyme were added to cell suspensions as described previously to a final concentration of 5,000 AU and 100 µg per ml, respectively. Cuvettes were incubated in an ice-bath for 5 min before electroporation. Electroporation was performed at 12.5 kV/cm at 25-µF capacitance with a single pulse. The samples were stored in an ice-bath prior to enumeration of CFU.

RESULTS AND DISCUSSION

Effect of UHP and EP on viability loss and sublethal injury

The CFU in cell suspensions of the three pathogens before and after UHP (50,000 lb/in^2 for 1 min) treatment was enumerated on both TSA and a selective medium to determine the levels of viability loss and sublethal injury among the survivors (Table 1, all results are presented in $\log_{10}$ units [U] and each item of data is an average of two to four separate counts). Viability loss or cell death estimated from the CFU on TSA before and after the treatments ranged from 3.7 to 6.9 $\log_{10}$ U by UHP and 2.8 to 4.4 $\log_{10}$ U by EP. Before the treat- ments, the numbers of CFU on the selective media were lower than the corresponding numbers of CFU on TSA and differences ranged from 0.1 (for L. monocytogenes) to 2.0 (for S. typhimurium) $\log_{10}$ U. The lower CFU counts on selective media could be due to inherent sensitivity of the strains to the selective compounds in the media and/or due to stress imposed during preparation and holding of cell suspensions at 4°C prior to enumeration (20 and 21). After treatment, the differences in counts between selective and nonselective media ranged from 3.0 to 6.5 $\log_{10}$ U by UHP and from 0.3 to 2.3 $\log_{10}$ U by EP. The inability of some of the cells surviving a treatment to form colonies on a selective medium while retaining the ability to form colonies on a nonselective medium is a manifestation of sublethal injury and results from the inability of these injured cells to multiply in the selective

Table 1. Effect of UHP and EP treatment on viability loss and sublethal injury

Treatment[a] and bacterial strain	Enumeration time	Concn of cells ($\log_{10}$ CFU/ml) In medium[b] Nonselective	Selective	Dead & injured After treatment[c] ($\log_{10}$ CFU/ml)
UHP				
L. monocytogenes Scott A	Before UHP	11.9	11.8	
	After UHP	5.0	2.0	6.9 and 3.0
E. coli O157:H7 932	Before UHP	11.7	11.2	
	After UHP	8.0	5.0	3.7 and 3.0
S. typhimurium M1	Before UHP	13.4	11.9	
	After UHP	8.5	2.0	4.9 and 6.5
EP				
L. monocytogenes Scott A	Before EP	13.8	13.5	
	After EP	11.0	10.7	2.8 and 0.3
E. coli O157:H7 932	Before EP	13.4	13.2	
	After EP	9.0	7.7	4.4 and 1.3
S. typhimurium M1	Before EP	12.9	10.9	
	After EP	8.6	6.3	4.3 and 2.3

[a] The cells were subjected to 50,000 lb/in^2 for 1 min. The times to attain the increase from 14.7 to 50,000 lb/in^2 and drop to 14.7 lb/in^2 were about 23 and 4 min, respectively. Cells were subjected to 12.5 kV/cm at a 25-μF capacitance.

[b] The nonselective medium was TSA for all strains, and the selective media were modified Oxford medium for L. monocytogenes, violet red bile agar for E. coli, and xylose-lysine deoxycholate agar for S. typhimurium. Each item of data represents the mean of two to four readings.

[c] The differences in numbers of CFU in TSA before and after treatment were considered to indicate the number of dead cells, and the differences between TSA (nonselective) and selective agar after treatment were considered to indicate the number of injured cells (18).

environment (20 and 21). These cells subsequently die in the presence of selective agents (24). These results have demonstrated that both UHP and EP treatments not only inflict lethal but also inflict sublethal injury to gram-positive and gram-negative bacterial cells.

Effect of UHP treatment in combination with bacteriocins

Sublethally injured bacterial cells have been reported to become sensitive to bacteriocins of lactic acid bacteria (12, 24). To determine if the UHP treatment has increased bactericidal efficiency in the presence of bacteriocin(s), L. monocytogenes, E. coli and S. typhimurium were subjected to UHP in the presence of pediocin AcH, nisin, or their combination. UHP treatment alone resulted in the viability loss or cell death from 3.1 to 4.9 $\log_{10}$ U for L. monocytogenes, 1.7 to 4.8 $\log_{10}$ U for E. coli, and 3.3 to 4.9 $\log_{10}$ U for S. typhimurium as the pressure increased from 30,000 to 50,000 lb/in^2 (Table 2). However, in the presence of bacteriocins, either individually or in combination, a greater reduction in viability occurred. The highest reductions with UHP (50,000 lb/in^2) and bacteriocins were as follows: 9.3 $\log_{10}$ U for L. monocytogenes with the pediocin AcH and nisin combination, and 8.4 $\log_{10}$ U for E. coli as well as 9.9 $\log_{10}$ U for S. typhimurium with nisin.

Table 2. Effect of UHP treatment in combination with bacteriocin(s)

Bacterial strain	Treatment[a]	Conc of cells ($\log_{10}$ CFU/ml) at[b]		
		30,000 lb/in^2	40,000 lb/in^2	50,000 lb/in^2
L. monocytogenes Scott A	Control[c]	12.5	12.5	12.7
	UHP	9.4	8.9	7.8
	UHP + Ped	7.9	7.7	6.0
	UHP + Nis	8.9	8.1	4.5
	UHP + Ped + Nis	7.8	7.1	3.4
E. coli O157:H7 932	Control	12.8	12.8	14.1
	UHP	11.1	10.7	9.3
	UHP + Ped	10.3	9.9	7.5
	UHP + Nis	8.9	8.7	5.7
	UHP + Ped + Nis	9.7	9.6	6.8
S. typhimurium M1	Control	13.9	13.9	13.9
	UHP	10.6	9.7	7.6
	UHP + Ped	9.8	8.1	5.5
	UHP + Nis	9.7	6.8	4.0
	UHP + Ped + Nis	9.8	8.0	5.6

[a] Ped, pediocin AcH; Nis, Nisin. The bacteriocins were used separetely or in combination at a final concentration of 5,000 AU/ml.

[b] The pressures were applied for about 1 min. The times to attain highest pressure from 14.7 lb/in^2 and then drop to 14.7 lb/in^2 were, respectively, 7 and 3 min for 30,000, 10 and 4 min for 40,000, and 20 and 9 min for 50,000 lb/in^2. Each item of data is the mean of four readings.

[c] Controls were CFU per milliliter prior to treatment. The difference in the numbers of CFU before and after a treatment was used to determine viability loss or cell death.

Table 3. Effect of EP treatment in combination with bacteriocin(s)

Bacterial strain	Treatment[a]	Concn of cells ($\log_{10}$ CFU/ml)[b]
L. monocytogenes Scott A	Control	13.7
	EP	11.0
	EP + Ped	10.5
	EP + Nis	7.8
	EP + Ped + Nis	8.2
E. coli O157:H7 932	Control	12.0
	EP	8.5
	EP + Ped	8.2
	EP + Nis	7.8
	EP + Ped + Nis	7.9
S. typhimurium M1	Control	11.7
	EP	8.6
	EP + Ped	8.0
	EP + Nis	7.9
	EP + Ped + Nis	8.0

[a] For explanation, see footnote a in Table 2. EP was applied at 12.5 kV and a 25-μF capacitance in a single pulse.

[b] CFU per milliliter before treatment. Each item of the data is the mean of two readings.

Effect of EP treatment in combination with bacteriocins

As in UHP, EP treatment alone reduced the viability of three pathogens as follows: 2.7 $\log_{10}$ U for *L. monocytogenes*, 3.5 $\log_{10}$ U for *E. coli*, and 3.1 $\log_{10}$ U for *S. typhimurium* (Table 3). The differential killing effect by single-pulse EP treatment is commonly observed from strain to strain. Furthermore, size of cells is one of the major factors in inflicting damage to cells by electroporation (26). In this study, *L. monocytogenes* has the smallest size as compared to both *E. coli* and *S. typhimurium* (16, 25). The smaller the size the higher the field strength needed for killing. In the presence of bacteriocin(s), the greatest reductions were by EP + nisin, 5.9 $\log_{10}$ U for *L. monocytogenes*, 4.2 $\log_{10}$ U for *E. coli*, and 3.8 $\log_{10}$ U for *S. typhimurium* (Table 3). The other bacteriocin, pediocin, had less of an effect when combined with EP than nisin.

Under the test conditions used in this study, the bactericidal efficiency was greater in the UHP treatment than in the single-pulse EP treatment, even with 30,000 lb/in^2 for 1 min, and viability losses were greater when bacteriocins and lysozyme were present in the UHP than the EP treatments. In a previous study with bacteriocins, we proposed that pediocin AcH and nisin, in combination, could have increased antibacterial efficiency against gram-positive bacteria (7). In that study, we also saw a similar effect with UHP-treated but not EP-treated, *L. monocytogenes*. For the two gram-negative strains, nisin alone, however, produced the greatest effect.

In the present study, very large cell populations but relatively low bacteriocin concentrations were used. For an ideal condition to study the possible additive effects for bacteriocins, one needs to consider the pH and ionic environment of the suspending medium and relative cell and bacteriocin concentrations. One of the bacteriocins should be at a concentration to produce its maximum bactericidal effect in a population. In the present study, these condition were different from those in the previous study at atmospheric pressures (7).

Effect of UHP treatment in combination with bacteriocins and lysozyme on spoilage bacteria

Previous studies have indicated that the combination of UHP and bacteriocins had a greater ability to reduce the population of three pathogens. In this study, lysozyme was included to determine if lysozyme had more lethal effect on spoilage bacteria. Leuconostoc strain 03 (gram-positive) and S. liquefaciens Lm (gram-negative) both isolated from spoiled processed meat were used in this study.

The UHP alone at 30,000 lb/in^2 reduced the viability of Leuconostoc strain 03 and S. liquefaciens Lm by 2.5 and 4.2 $\log_{10}$ U, respectively (Table 4). The viability loss of Leuconostoc and S. liquefaciens Lm increased respectively to 5.3 and 4.9 $\log_{10}$ U by subjecting them to UHP in the presence of bacteriocins.

Table 4. Effect of UHP treatment in combination with bacteriocins and lysozyme on spoilage bacteria

Bacterial strain	Treatment[a]	$\log_{10}$CFU/ml[b]	Dead cells[c]
Leuconostoc O3	Control	14.1	
	UHP	11.6	2.5
	UHP + Ped + Nis	8.8	5.3
	UHP + Ped + Nis + Lysz	4.8	9.3
S. liquefaciens	Control	12.1	
	UHP	7.9	4.2
	UHP + Ped + Nis	7.2	4.9
	UHP + Ped + Nis + Lysz	6.7	5.4

[a] Controls were cells without the UHP treatment. The pressure were applied a 30,000 lb/in^2 for 1 min. The time to attain the highest pressure from 14.7 lb/in^2 and then drop to 14.7 lb/in^2 were, respectively, 7 and 4 min for Leuconostoc and 8 and 4 min for S. liquefaciens. Ped, pediocin AcH; Nis, nisin; Lysz, lysozyme. The bacteriocins were used in combination at a final concentration of 5,000 AU/ml and a final concentration of lysozyme was 100 µg/ml.

[b] CFU per milliliter of each strain on either MRS or TSA agar. Each item of the data is the mean of four readings.

[c] The differences in the numbers of CFU before and after atreatment was used to determine viability loss or cell death.

The addition of lysozyme to the UHP bacteriocin combination had the greatest effect on viability loss (9.3 and 5.4 $\log_{10}$ U reduction) for the two spoilage bacteria, Leuconostoc and S. liquefaciens Lm, respectively.

Effect of UHP treatment in combination with bacteriocins and lysozyme on viability loss of pathogenic bacteria

Two pathogens, L. monocytogenes Scott A (gram-positive) and S. typhimurium (gram-negative) were subjected to the UHP treatment in the presence of bacteriocins and lysozyme (Table 5). When both bacterial strains were subjected to UHP alone, the viability loss was 2.8 log U. A higher viability loss was found when both strains were subjected to UHP along with bacteriocins. Bacteriocins enhanced the viability loss when combined with UHP and was greatest for LM. The UHP treatment in the presence of bacteriocins and the addition of lysozyme had a dramatic enhanced viability loss, especially for LM (13.8 log kill), and also for ST (6.3 log kill).

Effect of EP treatment in combination with bacteriocins and lysozyme

Two gram-negative (E. coli and S. liquefaciens) and one gram-positive (Leuconostoc 03) bacterial strains were used in this study. Bacterial suspensions were electroporated in the presence of bacteriocins and lysozyme.

Table 5. Effect of UHP treatment in combination with bacteriocins and lysozyme on two pathogenic bacteria

Bacterial strain	Treatment[a]	$\log_{10}$CFU/ml[b]	Dead cells[c]
L. monocytogenes Scott A	Control	14.8	
	UHP	12.0	2.8
	UHP + Ped + Nis	10.1	4.7
	UHP + Ped + Nis + Lys	1.0	13.8
S. typhimurium M1	Control	11.6	
	UHP	8.8	2.8
	UHP + Ped + Nis	7.5	4.1
	UHP + Ped + Nis + Lysz	5.3	6.3

[a] Controls were cells without the UHP treatment. The pressure were applied at 30,000 lb/in^2 for 1 min. The time to attain the highest pressure from 14.7 lb/in^2 and then drop to 14.7 lb/in^2 were, respectively, 7 and 4 min for L. monocytogenes and 8 and 4 min for S. typhimurium. Ped, pediocin AcH; Nis, nisin; Lysz, lysozyme. The bacteriocins were used in combination at a final concentration of 5,000 AU/ml and a final concentration of lysozyme was 100 μg/ml.

[b] CFU per milliliter of each strain on TSA agar. Each item of the data is the mean of four readings.

[c] The differences in the log numbers of CFU/ml before and after a treatment was used to determine viability loss or cell death.

The viability losses of three strains were as follows: 11.5 $\log_{10}$ U for Leuconostoc 03, 7.3 $\log_{10}$ U for S. liquefaciens, and 6.4 $\log_{10}$ U for E. coli compared to controls (Table 6).

In both UHP and electroporation, lysozyme greatly enhanced the bactericidal effect on gram-positive spoilage and pathogenic bacteria. This has to due with the hydrolysis of the β-1,4-glycosidic bond in the peptidoglycan found in gram-positive bacteria (6).

CONCLUSIONS

The results presented here have shown that both UHP and EP treatments caused viability loss and sublethal injury to cells of the bacteria tested. The degree of viability loss and sublethal injury, however, varied from strain to strain and treatment. Because of the sensitivity of injured cells to bacteriocin(s) and/or lysozyme, an increase in viability loss (or cell death) occurs when UHP or EP treatment is given in the presence of bacteriocin(s) and lysozyme. Thus, nonthermal treatments such as UHP and EP, in combination with biopreservatives, bacteriocins of food grade lactic acid bacteria and lysozyme, can be used to increase bactericidal efficiency and enhance the safety and shelf life of foods.

Table 6. Effect of EP treatment in combination with bacteriocin(s) and lysozyme me on spoilage and pathogenic bacteria

Bacterial strain	Treatment[a]	$\log_{10}$CFU/ml[b]	Dead cells[c]
Leuconostoc 03	Control	14.0	
	EP + Ped + Nis + Lysz	2.5	11.5
S. liquefaciens	Control	14.5	
	EP + Ped + Nis + Lysz	7.2	7.3
E. coli O157:H7 932	Control	13.0	
	EP + Ped + Nis + Lysz	6.6	6.4

[a] Controls were cells without EP treatment. EP was applied at 12.5 kV and 25-μF capacitance in a single pulse. Ped, pediocin AcH; Nis, nisin; Lysz,lysozyme. The bacteriocins were used at a final concentration of 5,000 AU/ml and a final concentration of lysozyme was 100 μg/ml.

[b] CFU per milliliter of each strain on either MRS or TSA agar. Each item of the data is the mean of four readings.

[c] The differences in the numbers of CFU before and after atreatment was used to determine viability loss or cell death.

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